# HONEY CATALASE: OCCURRENCE AND SOME KINETIC PROPERTIES

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SUMMARY

There have been earlier claims of the existence of a catalase in honey, but these were based on inconclusive experiments and inappropriate methods. In the present study, a correlation of manometric and spectrophotometric findings has provided the first unequivocal evidence for the occurrence of this enzyme in honey.

An investigation of some of the kinetic properties of the honey catalase revealed a *p*H optimum at 7–8·5 and a substrate concentration optimum at 0·018M. The Michaelis constant was 0.0154M. The effect of enzyme concentration on reaction velocity was linear. The reaction followed a first order, being dependent on the $H_2O_2$ concentration.

In honey, catalase apparently serves as a control on the $H_2O_2$ equilibrium, thus regulating the activity of the honey glucose oxidase.

## INTRODUCTION

Auzinger (1910*a*,*b*) was the first to report the presence of a catalase in honey. This was based on the fact that a gas was evolved and could be collected in a fermentation apparatus over a period of 15–24 hours when a mixture of honey and $H_2O_2$ was introduced into the system. He failed to show that this gas was derived from the $H_2O_2$ or that the $H_2O_2$ had actually been decomposed, whether by an enzyme or other means. Indeed, it appeared more likely that the evolution of a gas over such an extended period was due to bacterial fermentation or, at best, to a non-enzymatic reduction of the peroxide.

Since that time, a number of other workers (Moreau, 1911; Gothe, 1914; Stitz, 1930; Gillette, 1931; Kiermeier & Köberlein, 1954; Fedotova, 1957; Dzialoszynski & Kuik, 1963; Gasenko & Alekseev, 1963) have reported the presence of a catalase in certain, but not all, honey samples. Some of the methods used (Moreau, 1911; Gothe, 1914; Gillette, 1931) were similar to Auzinger's gasometric technique and subject to the same criticism. Disappearance of $H_2O_2$ was followed by titration with $KMnO_4$ in two reports (Kiermeier & Köberlein, 1954; Fedotova, 1957), and by titration with iodine in KI in another (Dzialoszynski & Kuik, 1963). Although these are standard methods for $H_2O_2$, they are entirely inappropriate for honey, since both reagents are known to react with carbohydrate materials (Pigman & Goepp, 1948) and have likewise been shown to react with honey (Griebel & Hess, 1940; Schepartz & Subers, 1966). Two of the above reports did not mention the methods used to determine the catalase (Stitz, 1930; Gasenko & Alekseev, 1963). Hence, up to the present, there has been no unequivocal demonstration of the presence of a catalase in honey.

While conducting experiments on a honey glucose oxidase (Schepartz & Subers, 1964), it was noted that in certain preparations some of the $H_2O_2$ produced by the action of this enzyme on glucose disappeared. Also, where extra $H_2O_2$ was added to the system, there was an unaccountable loss of significant quantities of $H_2O_2$. These findings prompted an investigation into the possible occurrence of a catalase in honey, the results of which are presented here.

## MATERIALS AND METHODS

The honey samples were obtained from commercial sources, were unheated, stored at 4°C., and identified as follows:

HS-36 clover (*Trifolium*)
HS-37 north-eastern fall-flower blend
HS-38 cotton (*Gossypium*)

Hydrogen peroxide was a 30% $H_2O_2$ solution obtained from J. T. Baker Chemical Co.[1], *o*-dianisidine (3,3′-dimethoxybenzidine) from Eastman Organic Chemicals, and peroxidase (Horseradish, Type I) from Sigma Chemical Co.

Standard Warburg procedures were employed in all manometric experiments. These were run in air at 37° and an oscillation rate of 150/min. on a Gilson Medical Electronics circular apparatus. The main-space of the vessels contained enzyme in buffer solution. The substrate was introduced from a side-arm sac. Alkali was present in the centre well.

In spectrophotometric experiments, enzyme samples in buffer solution were placed in test-tubes, substrate added to start the reaction, the tubes mixed and then incubated in a water bath at 37°. Samples of the reaction mixture were withdrawn at desired intervals and transferred to the assay system given below.

Spectrophotometric measurements were made on a Bausch and Lomb Spectronic 505 Recording Spectrophotometer. Stock $H_2O_2$ solutions were assayed by direct absorbance readings at 250 m$\mu$ (Beers & Sizer, 1952), where $\epsilon_{250} = 19{\cdot}25$. This type of measurement was not applicable to enzyme reaction mixtures, because of high background absorbance. Therefore, for the assay of $H_2O_2$ in reaction mixtures, the peroxidase-dianisidine system (Schepartz & Subers, 1964) was used, after calibration against the direct absorbance method. The standard test system consisted of the following in a total volume of 3·5 ml.: 3·2 ml. phosphate buffer (0·2M sodium phosphate, *p*H 6·1); 0·1 ml. *o*-dianisidine (3·5 mg./ml. in 95% ethanol); 1 drop peroxidase (0·04 mg./ml. in the phosphate buffer); 0·1 ml. $H_2O$; 0·1 ml. solution of unknown $H_2O_2$ concentration. After 5 minutes ($t_5$) at room temperature, 2 drops of concentrated HCl were added to shift the *p*H to 2. The absorbance was then read at 402 m$\mu$ between $t_{10}$ and $t_{20}$ minutes and compared with a blank containing $H_2O$ in place of $H_2O_2$. In this system the absorbance is linear from 0·2 to 2·8 $\mu$g. $H_2O_2$/ml., and the following relationship holds: absorbance $\times$ 3·64 = amount of $H_2O_2$ in $\mu$g./ml.

Protein was determined by the spectral method of Warburg and Christian (1942) as modified by Layne (1957).

[1] Mention of company or trade names does not imply endorsement by the Department over others of a similar nature not named.

Dialyses were all conducted for 24 hours against running tap-water (approximately 22°).

In all these experiments 37° was used as incubation temperature because of the very low level of activity at the usual reaction temperatures for catalase (0–20°).

One unit of honey catalase was defined as that amount of enzyme catalysing the decomposition of 1 $\mu$ mole of $H_2O_2$ per minute at 37°, optimum *p*H (7–8·5), and optimum substrate concentration (0·018M).

## RESULTS AND DISCUSSION

### *Occurrence of the enzyme*

A honey sample (HS-38) was dissolved in phosphate buffer and studied manometrically at two peroxide levels (Fig. 1). During the first 20–30 minutes it

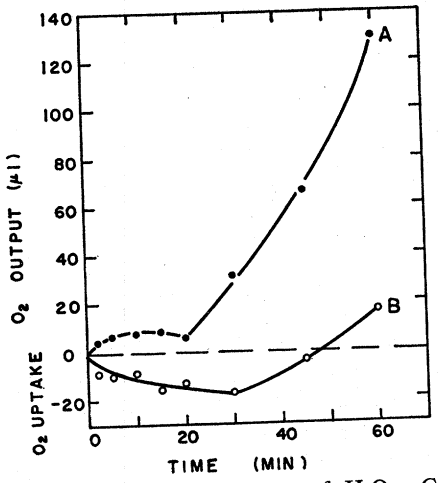


FIG. 1. Manometric response of honey in presence of $H_2O_2$. Complete system contained 3·3 ml. honey solution (10 g. honey HS-38 and 10 ml. M/15 sodium phosphate, *p*H 6·9) in main-space; 0·2 ml. $H_2O_2$ solution (containing 8300 $\mu$g. in A, 1600 $\mu$g. in B) in side-arm sac; 0·2 ml. 10% KOH in centre well. Blanks were run without honey, without substrate.

would appear that there is a competition between the glucose oxidase system known to be present (Schepartz & Subers, 1964) and a catalase system according to the reactions:

$$\text{glucose (in honey)} + O_2 \xrightarrow{\text{glucose-oxidase}} \text{gluconolactone} + H_2O_2$$

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2$$

At the end of this period the activity of the glucose oxidase declines (Schepartz, 1965), and the catalase reaction progresses as indicated by the sharp increase in $O_2$ output. In the absence of glucose oxidase activity there is only an $O_2$ output due to the catalase. This is illustrated in Fig. 2, where a dialysed honey has been used as the enzyme source, with no honey glucose present. Proof that the $O_2$ output was truly due to an enzymatic degradation of the $H_2O_2$ is provided by the inactivity of a heated enzyme solution (Fig. 2).

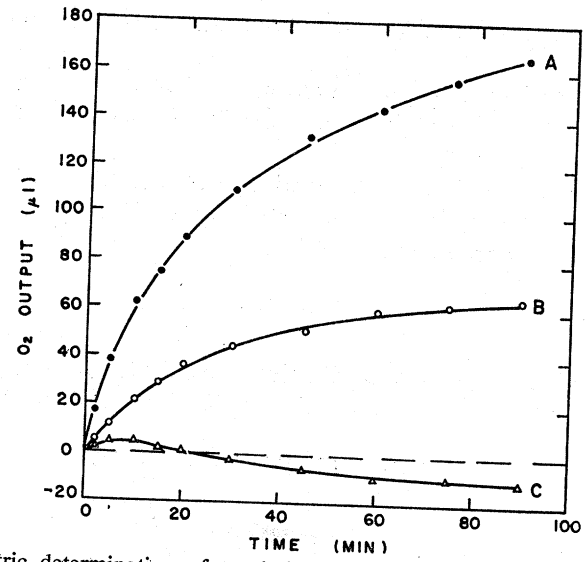


FIG. 2. Manometric determination of catalatic activity of a dialysed honey solution. Complete system contained 3·0 ml. dialysed honey solution (10 g. honey HS-38 dialysed against tap-$H_2O$, then diluted to 26 ml.) and 0·2 ml. 0·2M sodium phosphate, *p*H 6·9, in main-space; 0·3 ml. $H_2O_2$ solution (containing 8300 µg. in A and C, 1600 µg. in B) in side-arm sac; 0·2 ml. 10% KOH in centre well. In C, honey solution was heated for 5 min. at 100°. Blanks were run without honey, without substrate.

In similar experiments in which dialysed honey solutions were used as the enzyme source, the course of the reaction was followed by determining the disappearance of $H_2O_2$ with the peroxidase-dianisidine assay system. An example of the progress curves is shown in Fig. 3; their parabolic shape (obtained by either method) indicates the reaction is of a first order, being dependent on the concentration of $H_2O_2$ and that the equation $k = (\ln x_o/x)/t$ should hold. Fig. 4, where $\ln x_o/x$ plotted against $t$ gives a straight line, shows that this is so.

The catalatic activity of three different types of honey were compared manometrically and spectrophotometrically. Ten grams each of HS-36, -37 and -38 (see Materials and Methods) were dissolved in $H_2O$, dialysed and diluted to 25 ml. The $O_2$ output was then measured manometrically, using equal amounts of $H_2O_2$ as substrate for all three. The results are shown in Fig. 5. At the end of the manometric run, samples were removed from each flask and the amount of residual $H_2O_2$ determined in the peroxidase-dianisidine assay system. These values are fairly consistent with those obtained by extrapolating the manometric curves to the stopping time of the $H_2O_2$ assay, considering the amount of handling, dilutions, and the time involved (Table 1).

Immediately after the manometric experiment, the same enzyme solutions were used in a spectrophotometric experiment, the disappearance of the substrate again being followed by the peroxidase-dianisidine assay. Since the sensitivity of this test system requires only very low concentrations of $H_2O_2$, the catalase reaction had to be run at a substrate level of 0·003 M (385 µg./tube), which was only about one-sixth that used manometrically. This experiment was therefore not *directly* comparable with the previous one. However, if the data are plotted

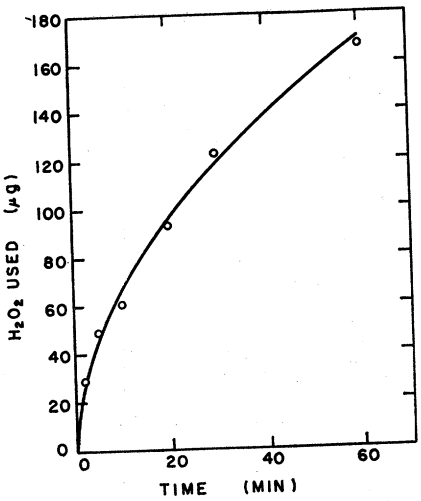


FIG. 3. Disappearance of $H_2O_2$ measured spectrophotometrically. Reaction system contained 3·0 ml. dialysed honey solution (10 g. HS-38 dialysed, then diluted to 25 ml.), 0·4 ml. 0·6M sodium phosphate, *p*H 7·0, and 0·1 ml. $H_2O_2$ (containing 805 μg.) added at $t_0$. Blanks were run without honey, without substrate. Samples were withdrawn at intervals shown and assayed for $H_2O_2$ in the peroxidase-dianisidine system (see Materials and Methods).

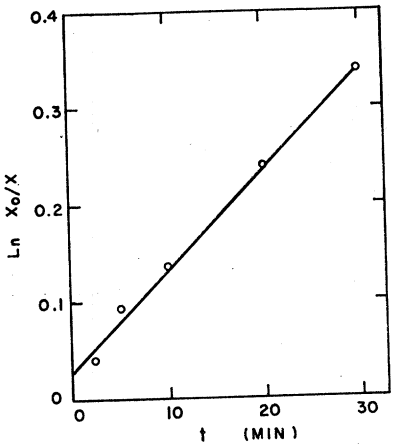


FIG. 4. Demonstration that the reaction in Fig. 3 is a first-order one; $x_0$ is the initial amount of $H_2O_2$, and $x$ the amount at time $t$.

TABLE 1. Relation between $H_2O_2$ disappearance and $O_2$ output. Conditions of the experiment were those given in Fig. 5

| *Honey sample* | HS-36 | HS-37 | HS-38 |
|---|---|---|---|
| Initial $H_2O_2$ (μg.) | 2440 | 2440 | 2440 |
| $H_2O_2$ used (μg.) | 0 | 460 | 230 |
| $O_2$ output (μl.) calculated* | 0 | 152 | 76 |
| $O_2$ output (μl.) found† | 2 | 140 | 48 |

* calculated from $H_2O_2$ used

† by extrapolation to $t_{75}$, Fig. 5

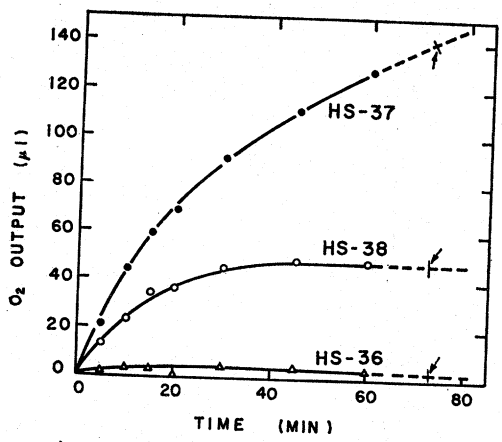


FIG. 5. Manometric comparison of three different types of honey. Each flask contained 3·0 ml. honey solution (10 g. HS-36, -37 or -38, dialysed and diluted as in Fig. 3), 0·4 ml. 0·6-M sodium phosphate, *p*H 7·0, in the main-space; 0·1 ml. $H_2O_2$ solution (containing 2440 μg.) in side-arm sac; 0·2 ml. 10% KOH in centre well. Blanks were run without honey, without substrate. Arrows indicate extrapolations to $t_{73}$, the stopping time for the $H_2O_2$ assays.

on the basis of a first-order reaction ($\ln x_o/x$ against $t$), the rate constants ($k$) can be obtained from the slopes of the lines (as in Fig. 4). When these constants are plotted against the initial velocities obtained in the manometric experiment, a direct relationship is found to exist (Fig. 6). This indicates that the $O_2$ output measured manometrically is *directly* related to the disappearance of $H_2O_2$ measured spectrophotometrically and, from this graph, $k = 3{\cdot}7 \times 10^{-3}v$. This is ample evidence that a catalase exists in honey and that its activity can be measured by either of these two methods.

It was established that catalase is a normal constituent of honey, and that the

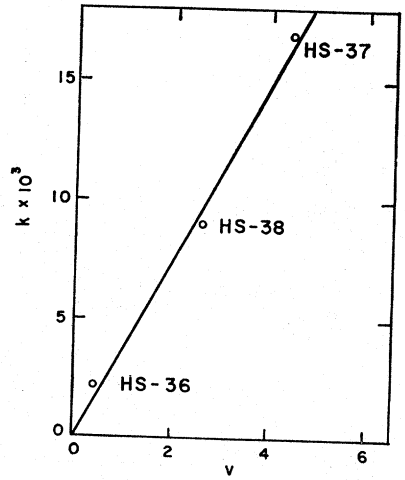


FIG. 6. Relationship between manometric and spectrophotometric data. Initial velocities ($v$) are from the experiment in Fig. 5. Values for $k$ are from a parallel experiment run spectrophotometrically. Conditions were same as in Fig. 3, except that 385 μg. $H_2O_2$ were used. The $k$ values were obtained from the slopes of the first-order lines, as in Fig. 4.

catalatic activity observed was not due to bacterial or fungal contamination, by microbiological counts on several samples of honey having significant catalase activity. The counts showed that contamination was rather low. Germination of organisms was so slow that the amount of catalase produced from such sources could not possibly have accounted for the level of activity found in the honey. This was confirmed by finding that pretreatment of the honey with an amount of merthiolate adequate for sterilization (12 mg./50 g. honey) did not affect the catalatic activity.

The occurrence of a catalase is not restricted to the three types of honey used in this study. Analysis of a variety of honeys has shown significant catalatic activity in a majority of samples examined (Schepartz & Subers, 1966).

*Kinetic properties of the enzyme*

(*a*) *Effect of* p*H*. The change in velocity with variation in *p*H was determined manometrically in 0·17-M sodium phosphate buffers from *p*H 3·3 to 10·7. As illustrated in Fig. 7, there was an optimum at *p*H 7-8·5. The optimum *p*H

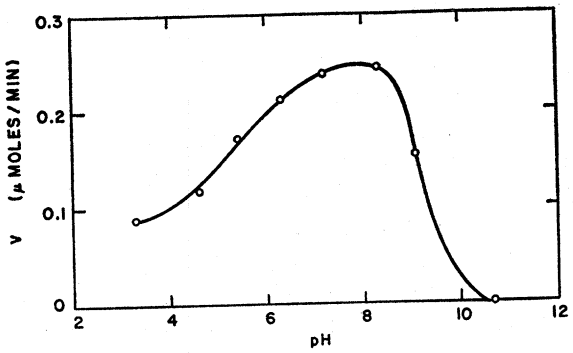


FIG. 7. Effect of *p*H on initial velocity. Complete system contained 2·4 ml. honey solution (40 g. HS-37 dialysed and diluted to 100 ml.), 1·0 ml. 0·6-M sodium phosphate at *p*H values from 3·3 to 10·7 in main-space; 0·1 ml. $H_2O_2$ solution (containing 2080 μg.) in side-arm sac; 0·2 ml. 10% KOH in centre well. Blanks were run without honey, without substrate.

range was relatively narrow, in contrast to data reported for other catalases, whose velocity of reaction was found constant over a range from about *p*H 3 to 9 (Chance & Maehly, 1961; Nicholls & Schonbaum, 1963).

(*b*) *Effect of enzyme concentration.* The effect of enzyme concentration on the initial velocity of reaction was studied manometrically over a six-fold range; the relationship was linear (Fig. 8). The fact that this line did not pass through the origin would suggest a slight inhibition or toxicity in the system (Dixon & Webb, 1958).

(*c*) *Effect of substrate concentration.* Using the manometric technique, a series of experiments was conducted in which the substrate concentration was varied over a wide range, keeping the enzyme concentration constant. There was an optimum at 0·018-M $H_2O_2$. The relation between the reciprocals of the initial velocity of reaction and the substrate concentration (Lineweaver & Burk, 1934) is shown in Fig. 9. The Michaelis constant ($K_m$) derived from this graph is 0·0154M.

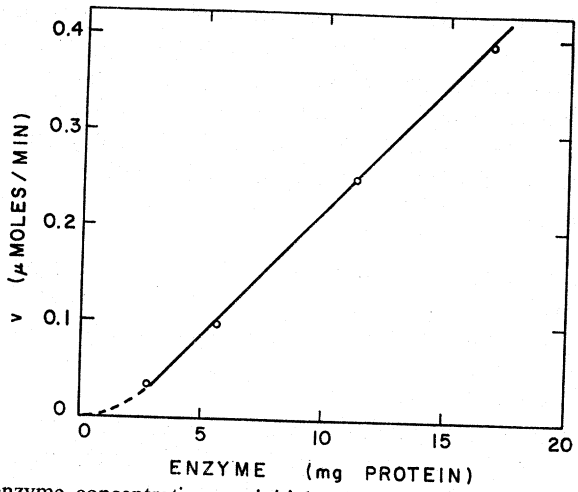


FIG. 8. Effect of enzyme concentration on initial velocity. Complete system contained honey solution (HS-37 prepared as in Fig. 7) in graded volumes from 0·5 to 3·0 ml., 0·4 ml. 0·6-M sodium phosphate, *p*H 7·0, $H_2O$ to give a total of 3·4 ml. in main-space; 0·1 ml. $H_2O_2$ solution (containing 2130 μg.) in side-arm sac; 0·2 ml. 10% KOH in centre well. Blanks were run without honey, without substrate.

The optimum substrate and $K_m$ values found here (where very dilute enzyme concentrations have been used) may have little significance, in the light of current thinking and the use of very high enzyme concentrations in studying catalase action (Bonnichsen *et al.*, 1947; Nicholls & Schonbaum, 1963). But it is interesting to note that the above value for $K_m$ compares well with values of 0·025M and 0·033M reported by von Euler and Josephson (1927) and Stern (1932) respectively.

Although it has been pointed out (Maehly & Chance, 1954) that catalases should be studied within the first few minutes if not seconds of reaction, this is

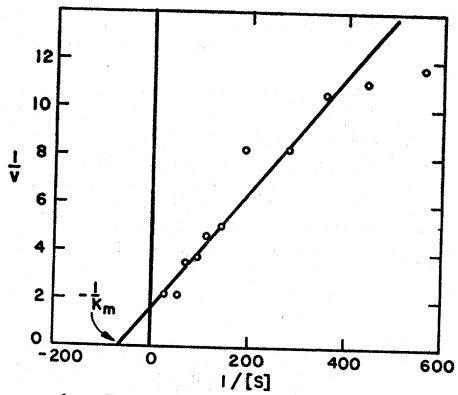


FIG. 9. Lineweaver-Burk graph. Complete system contained 3·0 ml. honey solution (HS-37 prepared as in Fig. 7), 0·3 ml. 0·6-M sodium phosphate, *p*H 7·5, in main-space; 0·2 ml. $H_2O_2$ solution (containing graduated amounts to give a final concentration range from 0·00179M to 0·0358M in side-arm sac; 0·2 ml. 10% KOH in centre well. Blanks were run without honey, without substrate. $v$ = initial velocity; $[s]$ = substrate concentration; $K_m$ = Michaelis constant.

not always possible. With honey catalase, for example, only a highly impure preparation of low activity is available, and only the slower methods can be utilized. Indeed, it is quite remarkable that, using nothing more refined than a dialysed honey solution as the enzyme source, considerable kinetic work could be performed, and what would appear to be rather clear-cut results obtained.

It is most unusual for both a catalase and a glucose oxidase to occur in the same natural material, as they do in honey. In these circumstances the catalase would appear to function as a control on the $H_2O_2$ equilibrium, and thus to regulate the activity of the oxidase.

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